EXHIBIT B

Improved organ size conferred by the RKS4 receptor

To study the function of RKS4 during development we have followed both gain- and loss-of-function approaches. Overexpression lines (RKS4-OX) were generated by ectopically expressing the *RKS4* full-length cDNA in *Arabidopsis* wild-type plants under the control of the CaMV 35S promoter and were studied along two T-DNA insertion lines *rks4-1* and *rks4-2* were analyzed in detail. The T-DNA insertion site was confirmed in both lines by PCR analysis and sequencing, which indicated that in *rks4-1* the T-DNA was inserted in intron 5 whereas in the case of *rks4-2* it was found in intron10 (Figure 1a). Nevertheless the T-DNA insertion still has led to the disruption of the *RKS4* gene in both lines since RT-PCR analysis on 10d seedlings showed that its full-length messenger was no longer present (Figure 1b, upper panel). However a truncated form of the messenger appeared to still be produced in both lines (Figure 1b, 2nd and 3rd panels), and at a high level in *rks4-1*, which may in fact lead to new functionality instead of a *sensus stricto* loss of function. Its effect on plant development is described hereafter.

Plating of RKS4-OX T_2 seed for selection revealed an increase in seed size that was visible with the naked eye in the case of one of the overexpression lines (OX1). Consequently seed length was measured for all lines (Figure 2f). This showed that seed size increase was in fact a characteristic of RKS4-OX lines, although the extent of the increase fluctuated probably as a result of differences in expression level. The largest increase, visible by eye, was 27.6% (RKS4-OX1). A second group of lines also distinguished itself by an increase around 15% (OX2, 5, 8 and 11), the rest was found around 10%. Differences were not further investigated in the present work but based on this information we chose to study RKS4-OX1 and –OX2 in more detail. Changes in *RKS4* steady state mRNA level were verified in these two lines by RT-PCR and confirmed that the *RKS4* gene is indeed overexpressed in RKS4-OX plants (Figure 1b). This also revealed that it is much more expressed in RKS4-OX2 than in RKS4-OX1.

Modifying *RKS4* expression levels appeared to clearly influence growth characteristics in both *rks4* and RKS4-OX plants. Our first visual observations had already revealed a strong effect of RKS4 overexpression on seed size, confirmed by seed length measurements. Seed weight determination also showed that this was accompanied by an even more dramatic increase in dry weight with 81.9% and 33.7% heavier seeds for RKS4-OX1 and -OX2, respectively (Figure 2f). In *rks4* lines seed length was only slightly decreased (5.2% and 3.5% for *rks4-1* and -2, respectively) and no significant change in seed weight was observed (Figure 2f). The limited effect of RKS4 "KO" on seed size can be explained by the fact that it is not normally expressed in the seed, expect for the few cells of the provascular tissue in embryos (Figure 3c). Detailed measurements of cotyledon size post germination showed that seed weight increase might be, at least partly, due to larger cotyledons. Total surface area in both *rks4* and RKS4-OX was indeed increased according to the very same trend (compare Figure 2e with Figure 2f). Interestingly in *rks4* lines, this is mostly due to an increase in cell division (15% for *rks4-1* and 10.7% for *rks4-2*) and a slight increase in cell elongation for *rks4-1* (13.1%). Whereas in overexpression lines cell division is significantly reduced (15.5% for RKS4-OX1 and 4.9% for RKS4-OX2), suggesting that RKS4 may have an inhibitory role on this process. Nevertheless cotyledons are even larger than those of *rks4* seedlings and this is caused by an overstimulation of

Improved organ size conferred by the RKS4 receptor

To study the function of RKS4 during development we have followed both gain- and loss-of-function approaches. Overexpression lines (RKS4-OX) were generated by ectopically expressing the *RKS4* full-length cDNA in *Arabidopsis* wild-type plants under the control of the CaMV 35S promoter and were studied along two T-DNA insertion lines *rks4-1* and *rks4-2* were analyzed in detail. The T-DNA insertion site was confirmed in both lines by PCR analysis and sequencing, which indicated that in *rks4-1* the T-DNA was inserted in intron 5 whereas in the case of *rks4-2* it was found in intron10 (Figure 1a). Nevertheless the T-DNA insertion still has led to the disruption of the *RKS4* gene in both lines since RT-PCR analysis on 10d seedlings showed that its full-length messenger was no longer present (Figure 1b, upper panel). However a truncated form of the messenger appeared to still be produced in both lines (Figure 1b, 2nd and 3rd panels), and at a high level in *rks4-1*, which may in fact lead to new functionality instead of a *sensus stricto* loss of function. Its effect on plant development is described hereafter.

Plating of RKS4-OX T_2 seed for selection revealed an increase in seed size that was visible with the naked eye in the case of one of the overexpression lines (OX1). Consequently seed length was measured for all lines (Figure 2f). This showed that seed size increase was in fact a characteristic of RKS4-OX lines, although the extent of the increase fluctuated probably as a result of differences in expression level. The largest increase, visible by eye, was 27.6% (RKS4-OX1). A second group of lines also distinguished itself by an increase around 15% (OX2, 5, 8 and 11), the rest was found around 10%. Differences were not further investigated in the present work but based on this information we chose to study RKS4-OX1 and –OX2 in more detail. Changes in *RKS4* steady state mRNA level were verified in these two lines by RT-PCR and confirmed that the *RKS4* gene is indeed overexpressed in RKS4-OX plants (Figure 1b). This also revealed that it is much more expressed in RKS4-OX2 than in RKS4-OX1.

Modifying *RKS4* expression levels appeared to clearly influence growth characteristics in both *rks4* and RKS4-OX plants. Our first visual observations had already revealed a strong effect of RKS4 overexpression on seed size, confirmed by seed length measurements. Seed weight determination also showed that this was accompanied by an even more dramatic increase in dry weight with 81.9% and 33.7% heavier seeds for RKS4-OX1 and -OX2, respectively (Figure 2f). In *rks4* lines seed length was only slightly decreased (5.2% and 3.5% for *rks4-1* and -2, respectively) and no significant change in seed weight was observed (Figure 2f). The limited effect of RKS4 "KO" on seed size can be explained by the fact that it is not normally expressed in the seed, expect for the few cells of the provascular tissue in embryos (Figure 3c). Detailed measurements of cotyledon size post germination showed that seed weight increase might be, at least partly, due to larger cotyledons. Total surface area in both *rks4* and RKS4-OX was indeed increased according to the very same trend (compare Figure 2e with Figure 2f). Interestingly in *rks4* lines, this is mostly due to an increase in cell division (15% for *rks4-1* and 10.7% for *rks4-2*) and a slight increase in cell elongation for *rks4-1* (13.1%). Whereas in overexpression lines cell division is significantly reduced (15.5% for RKS4-OX1 and 4.9% for RKS4-OX2), suggesting that RKS4 may have an inhibitory role on this process. Nevertheless cotyledons are even larger than those of *rks4* seedlings and this is caused by an overstimulation of

cell elongation (plus 91.3% and 43.9%, for RKS4-OX1 and –OX2 respectively), indicating that RKS4 enhances cell expansion. Interestingly cotyledon cells in *rks4-1* were also larger than those of the wild-type, suggesting that the mechanism by which RKS4 influences cell size might be dose-dependent. The fact that RKS4-OX2, which shows a very strong RKS4 expression, has an intermediate phenotype (both in cotyledons and seeds) is in line with this hypothesis implying the need for a specific level of RKS4 expression in order to keep cell elongation balanced. The large size increase observed in the cotyledons of RKS4-OX1 was also visible later in the size and shape of its rosette leaves, especially under short day conditions, giving extremely robust rosettes with rounder and broader leaves (Figure 2c-d). RKS4-OX1 plants were more vigorous in general and also gave very large flowers (Figure 2a), in which the size of all organs seems to be increased. We performed a quantitative analysis of petal size (figure 2b) that showed that size increase here (plus 60%) was due to both cell elongation (plus 37.6%) and proliferation (plus 16.3%). One should keep in mind however that RKS4 is normally not expressed in petals and that neither RKS4-OX2 nor *rks4* plants have significantly altered petal sizes indicating that the effect we see here might be due to ectopic interactions. Altered expression of *RKS4* did not affect silique shape and size (data not shown), in spite of the seed changes mentioned in Figure 2f. As expected from its expression pattern, altering *RKS4* expression levels also affected root development. Measuring roots of seedlings grown on vertical plates did indeed reveal that, as in cotyledons, root size/length was significantly increased both in the KO and the overexpression lines (Figure 2g). Root length was more enhanced in *rks4-1* than in *rks4-2* (74% vs. 65.9%) and RKS4-OX1 showed the largest increase of all (83.7%), besides RKS4-OX2 also showed an intermediate phenotype (52.7% longer roots). To investigate the nature of this length increase we made use of a *CYCB1.1::GUS* construct (pCDG) as a marker for mitotic activity (Colón-Carmona *et al.*, 1999). pCDG was crossed in RKS4-OX lines (Figure 2h) and quantitative analysis of the number of GUS-positive cells in root tips showed that cell division was dramatically reduced in RKS4-OX1, but was not significantly changed in RKS4-OX2 (Figure 2i), which is in agreement with the limited reduction in cell proliferation (4.9%) observed in cotyledons (Figure 2e). In spite of the 3-fold reduction in cell division observed in RKS4-OX1, root length is still increased by 84% indicating that as in cotyledons the size increase in roots is caused by an overstimulation of cell elongation.

The sum of these observations is in accordance with the *RKS4* promoter activity (Figure 3) and suggests that the RKS4 receptor is involved in maintaining the size of the organs in which it is expressed. The fact that an increase and a decrease in its expression both can lead to larger organs (except in seeds) suggests a requirement for a specific level of RKS4 receptor at an optimum keeping organ size normal. Although loss of function of the receptor did not give rise to phenotypes as dramatic as its overexpression, it is clear that in the RKS4 knockouts cell division is stimulated at least in cotyledons and most likely in roots as well. The opposite is observed in the same organs of overexpression plants, confirming that cell division could be repressed/maintained under a certain level by RKS4. This was not observed in petals, where overexpression of *RKS4* stimulated cell division as well as elongation. However RKS4 is normally not expressed in petals and we might be looking at a pleiotropic effect due to an ectopic interaction that might not represent the endogenous

function of the RKS4 receptor but that of a paralog normally present in these tissue. Interestingly, in line RKS4-OX2 that shows a stronger expression of RKS4 the phenotypes observed are milder than in the other overexpression line or even absent like in petals. This may indicate that a saturation level has been reached in the number of receptors produced leading to weaker effects.

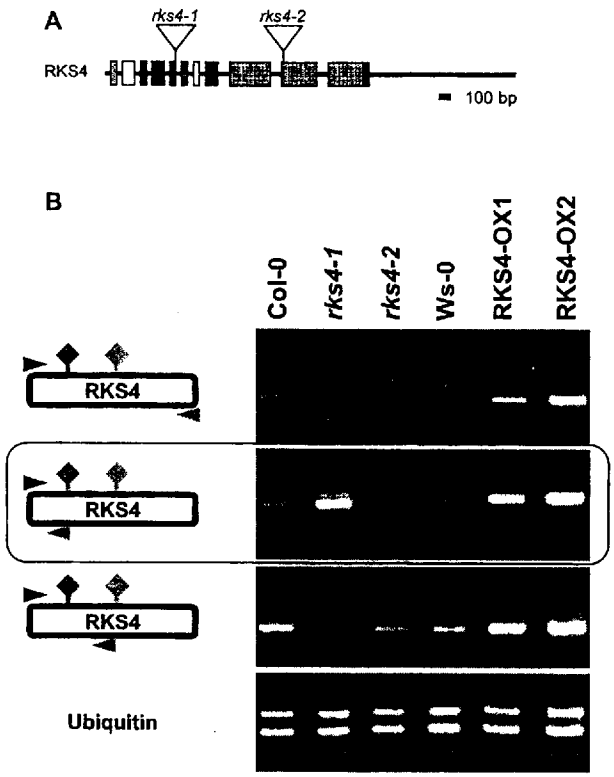

Figure 1. *RKS4* mRNA levels in knock-out and overexpression seedlings.

A. T-DNA insertion sites on the *RKS4* gene. **B.** RT-PCR analysis of the *RKS4* full-length messenger in 10 day-old seedlings from wild-type (Ws-0 and Col-0), two overexpression lines (RKS4-OX1 and RKS4-OX2) and two T-DNA insertion lines (*rks4-1* and *rks4-2*). A no template control (NTC) was included and equal amounts of cDNA template were assessed on the constitutive ubiquitin gene (Ubi). The position of the different oligonucleotides used within the RT-PCR reaction is indicated with respect to the different T-DNA integration sites on the right-hand side next to the corresponding Agarose Gel Electrophoresis pictures. Evidence for high expression of a truncated RKS4 messenger is shown in the second gel from the top (boxed).

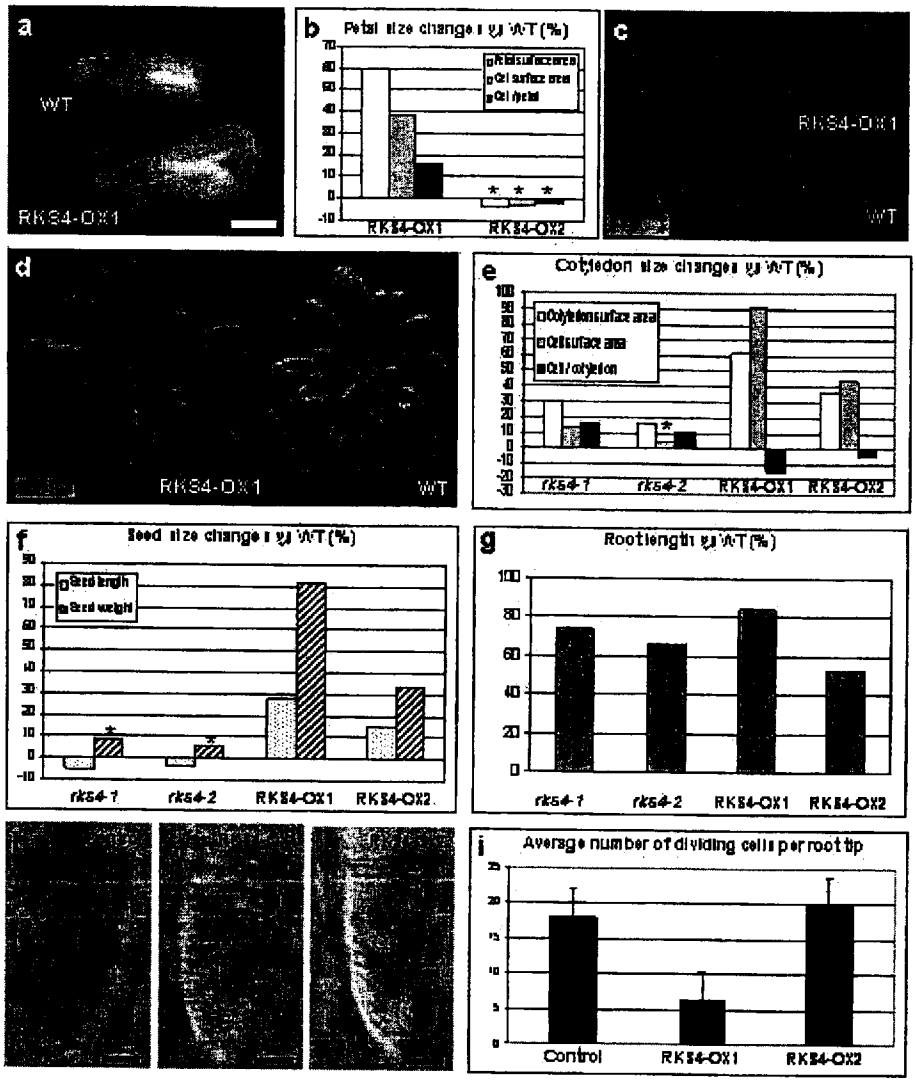

Figure 2. Morphological phenotypes induced by altered expression of RKS4.

Histograms shown in panels (b), (e), (f) and (g) are based on measurements performed on plants with RKS4 altered expression and depict changes in percentages related to the corresponding wild-type (Col-0 for rks4-1 and -2; Ws-0 for RKS4-OX1 and 2). Statistical significance of the observed differences was analyzed by t-test and the * indicates that the measured differences are not statistically significant (i.e. p-value > 0.05).

(a) Increased flower size due to RKS4 overexpression (RKS4-OX1) versus wild-type Ws-0 (WT) (scale bar = 1 mm).

(b) Influence of RKS4 overexpression on petal and petal epidermis cell size. The number of cells/petal was obtained by dividing the mean of the petal surface area by the mean of the cell surface area.

(c) Altered leaf shape in rosettes of RKS4-OX1 plants (scale bar in cm).

(d) Overview of rosette shape and size in RKS4-OX1 and WT plants (scale bar in cm).

(e) Influence of RKS4 altered expression on cotyledon size based on measurements of the surface area of cotyledons and of their palisade mesophyll cells. The number of cells per cotyledon was obtained by dividing the mean surface area of the cotyledons by the one of the mesophyll cells.

(f) Influence of RKS4 altered expression on seed yield determined by seed length and weight measurement.

(g) Influence of RKS4 altered expression on root length as measured on 9 day-old seedlings grown on vertical plates.

(h-i) Changes in root tip mitotic activity caused by overexpression of RKS4. (h) From left to right: GUS positive/dividing cells in the root tip of a 7-d old seedling containing the pCDG construct (Colón-Carmona, A., You, R., Haimovitch-Gal, T. and Peter Doerner, O. (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J. 20, 503-508) alone; reduced number of dividing cells in the root tip of a 7-d old F1 seedling from a cross between RKS4-OX1 and pCDG; root tip of a 7-d old F1 seedling from a cross between RKS4-OX2 and pCDG (scale bar = 50 um). (i) Histogram of the average number of GUS positive cells per root tip in the main root (standard deviation indicated by the error bars).

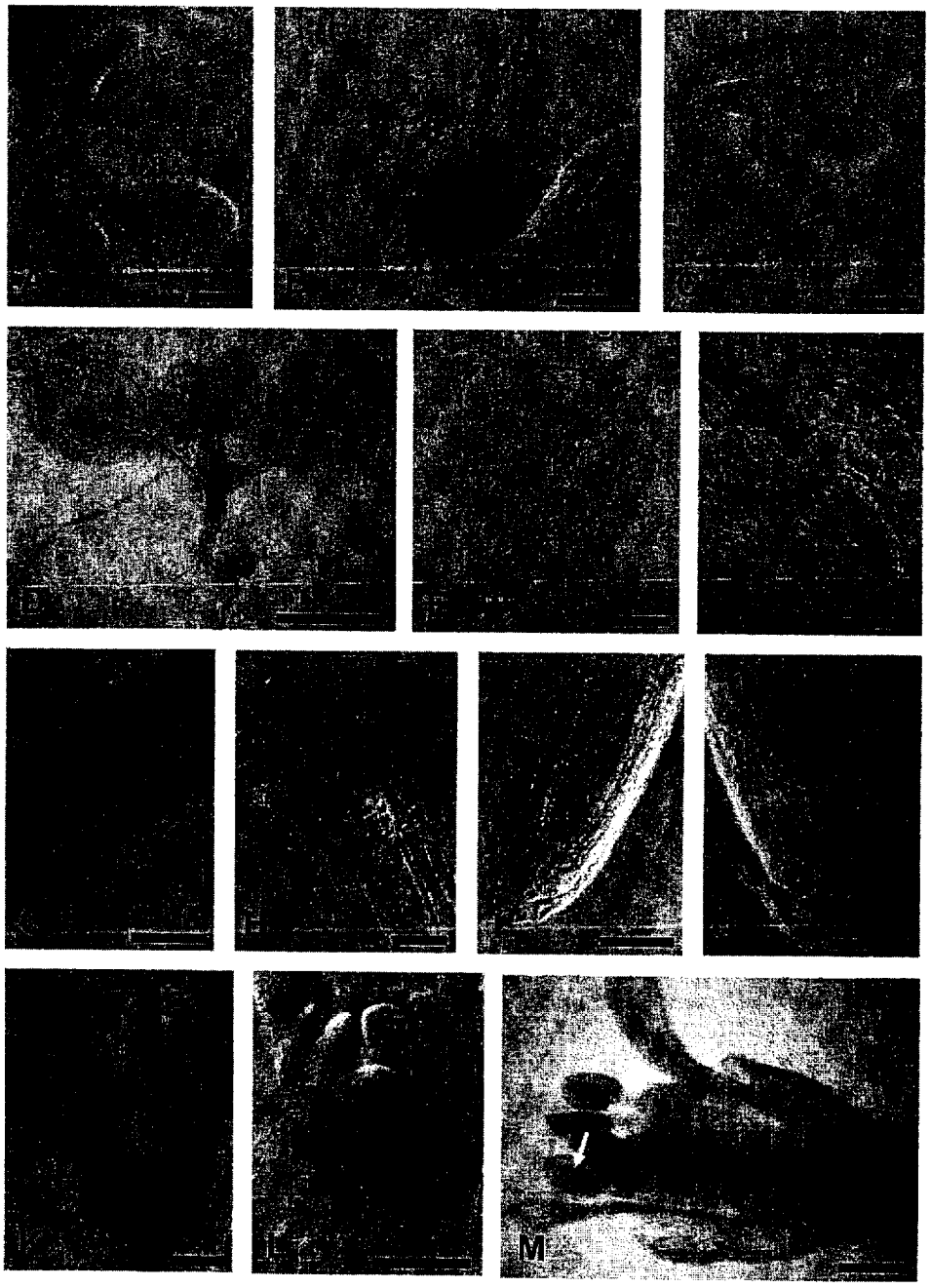

Figure 3. Histochemical localization of the *RKS4* promoter activity.

(a) Young developing seeds. GUS staining is localized in the funiculus and the chalaza (scale bar = 100 μm).

(b) Detailed view of the funiculus and the chalaza of a seed at the globular stage. The embryo (e) is in a different focus plane (scale bar = 50 μm).

(c) Dissected bent cotyledon embryo with GUS staining in the provascular tissue (scale bar = 50 μm).

(d) Ten-day old seedling with GUS staining in the vascular tissue (scale bar = 2 mm).

(e) Detailed view of a cotyledon from the seedling shown in panel (d) (scale bar = 0.5 mm).

(f) Detailed view of the perivascular staining observed in a cotyledon from a 10-day old seedling (scale bar = 50 μm).

(g) GUS staining around the xylem of a leaf petiole (scale bar = 50 μm).

(h) Detailed view of a root with a lateral root primordium (arrow). GUS staining is observed in the pericycle or the procambium (scale bar = 100 μm).

(i) Root apical meristem showing GUS staining in the stele initials (scale bar = 50 μm).

(j) Detailed view of a root apical meristem (scale bar = 20 μm).

(k) Shoot apical meristem area with strong GUS staining in/around the leaf vascular tissue and under the meristem where the vasculature divides in two bundles. Staining is visible in the provascular tissue of the leaf primordial as well (scale bar = 100 μm).

(l) Detailed view of a shoot apical meristem with GUS staining right under (scale bar = 100 μm).

(m) Flower with GUS staining around the sepal vasculature (black arrow) and around the anther connective tissue (white arrow) (scale bar = 0.5 mm).